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CHARACTERIZATION OF AN OPIOID-LIKE HIBERNATION INDUCTION TRIGGER

ANNUAL REPORT

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A new autoperfusion multiorgan preparation has been developed in our laboratories in which the dogs are anesthetized and artificially ventilated. The heart, lungs, liver, pancreas, duodenum and both kidneys are removed *en bloc* while being perfused by the heart and oxygenated by the lungs. A respirator is used for ventilation. Fresh blood, electrolytes, antibiotics and other nutrients are infused by intravenous dripping. No anticoagulants or inotropic drugs are necessary. The heart continues to pump blood to all the organs, and the lungs oxygenate the blood. The liver keeps its normal biological functions. Electrolyte and water balances are maintained, and metabolic wastes are removed by the kidneys. This preparation preserves the natural anatomic, as well as physiologic, connections of the organs without foreign material and requires little intervention. In the original study, liver congestion reduced survival

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time to an average of 14.8 hours. However, when plasma from deeply hibernating woodchucks, containing a hibernation induction trigger (HIT), was infused into the multiorgan autoperfusion system, an average survival time of 44 hours was obtained under non-sterile conditions. Comparable multiorgan survival times (averaging 46 hours) were achieved when the autoperfusions system was infused with the delta opioid, DADLE, which mimics the natural biologic activity in hibernators of the winter hibernating plasma. Moreover, a single lung transplantation study suggested that HIT extended effective organ preservation time substantially and that the lung can be preserved for more than 30 hours in the multiorgan autoperfusion system, utilizing HIT-containing plasma while maintaining the capability of supporting the whole body.

The autoperfusion methodology had extended organ survival time, however, progressive organ failure would still occur at some time. Many factors can affect the overall survival time. Surgical trauma, ischemia during preservation, accumulation of toxic substances, and formation of circulating platelet and/or platelet neutrophil aggregates all have a negative effect on tissue survival. One feature that was a consistent finding in all of our studies was the progressive fall in the white cell and platelet counts during the course of the experiment. These two findings suggest the hypothesis that one reason for progressive organ system failure during the autoperfusion studies may result from embolization of platelet and neutrophil aggregates in the heart, lungs, liver, and kidney.

If the embolization of platelet and neutrophil aggregates causes progressive multiorgan system failure in the autoperfusion model and the survival of organs is augmented by the use of HIT-containing plasma, one could hypothesize that one effect of HIT is to affect platelet function. In the canine model platelets behaved normally prior to HIT administration with a dose-response relationship between the amount of adenosine diphosphate (ADP) added and the extent of aggregation. However, after HIT, even though the platelets aggregated normally in response to ADP, the platelets disaggregated shortly after ADP stimulated aggregation, despite the use of high doses of ADP. Our preliminary results suggest that one mechanism of prolonged organ survival following HIT administration may be a result of its ability to prevent or reverse cell aggregation.

Studies of HIT and DADLE, utilizing the multiorgan autoperfusion system preservation, may establish a role for these agents in treatment of multiorgan system failure, clinical organ preservation, trauma management, and related applications. Moreover, the extension of organ survival, utilizing the multiorgan system, may serve as a rapid assay for HIT-activity of resolved plasma fractions. (cont'd, cont'd previous page)

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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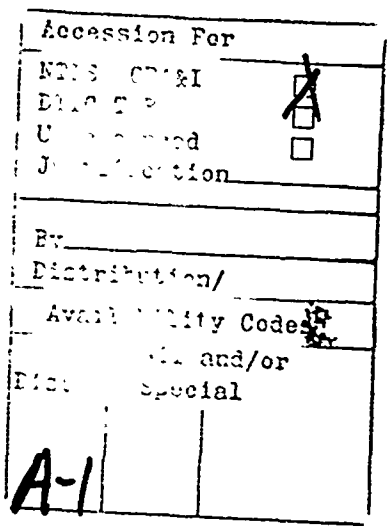

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INTRODUCTION

A hibernation induction trigger (HIT) molecule derived from the plasma of deeply hibernating woodchucks exerts profound effects mimicking a hibernation-like state when infused I.C.V. or I.V. in primates. The profound opiate-like behavioral and physiological depression occurring shortly after the infusion of the HIT-containing albumin fraction include hypothermia, bradycardia, long-term hypophagia and markedly depressed renal function. All of the aforementioned effects in primates are reversed or retarded by the infusion of the opiate antagonists, naloxone and naltrexone. (1-5) Such evidence enforces our hypothesis that not only is the HIT molecule not specific for hibernators, but that it may initiate its action through specific opioid receptor(s). We have now shown that naloxone, (6) the highly specific synthetic kappa agonist U69593, (7) and the naturally occurring kappa brain opioid agonist, dynorphin as well as the mu agonists, morphine and morphiceptin, can all block hibernation induced by HIT when infused via osmotic pumps which have been implanted subscapularly in summer-active ground squirrels (the traditional bioassay). Only the synthetic and more stable delta opioid D-Ala^2-Leu^5-Enkephalin (DADLE) induced hibernation in summer-active ground squirrels in a fashion similar to animals injected with HIT. (8-10) The latter experiments indicate that the delta opioid receptor and its ligand may mimic the action of the HIT molecule and may be intimately involved in natural winter and summer-induced hibernation. We now have preliminary evidence that the HIT-containing albumin fraction, but not the summer-active albumin fraction, can markedly reduce cell growth and thymidine incorporation in a cultured dog kidney cell line, indicating the HIT molecule has depressive effects on DNA synthesis. This rapid in vitro cell culture assay shows great promise in replacing the traditional seasonally restrictive ground squirrel bioassay for monitoring activity of more purified fractions thought to have HIT-activity. Our most recent experiments have shown that the HIT molecule as well as the delta opioid, DADLE, can also be used to dramatically extend the effective preservation time of a dog multiorgan autoperfusion system developed by Chien et al. In these breakthrough experiments, we have clearly shown that both HIT and DADLE can prolong multiorgan survival time for transplantation and/or organ preservation purposes by nearly three-fold. Utilizing this multiorgan autoperfusion system, we may be able to monitor the activity of more highly resolved fractions thought to have HIT-activity. We also have preliminary evidence that the HIT-molecule and possibly DADLE prevent platelet-neutrophil aggregates from forming and this may prolong organ survival by preventing the formation of micro thromboemboli throughout the vascular

system in these organs. If this mechanism of action of these molecules is confirmed, it could have profound practical clinical significance for all sorts of vascular and/or microvascular surgical procedures.

MATERIALS AND METHODS

A. SAMPLE PROCUREMENT

11 **Sample Procurement:** Plasma to be assayed for HIT activity will be obtained from woodchucks weighing 3.0 to 5.0 kg maintained in an 8' x 12' hibernaculum at 4-6°C and from hibernating and summer-active black bears maintained in enclosures with dens by Dr. Ralph Nelson at the University of Illinois, Urbana, IL. Blood from woodchucks will be drawn aseptically by heart ventricular puncture while these animals are in deep hibernation as evidenced by having a core temperature of approximately 5°C and heart rates of one to two beats per minute. Normally it takes these hibernators one or two hours to arouse (core temperature reaching 37°C and heart rate returning to normal) after this painless blood drawing procedure. Blood from black bears is obtained from the femoral artery after both active and hibernating animals are anesthetized with ketamine and Rompum. The bears' core temperature drops only 2 or 3°C even when hibernating and arousal can occur in minutes with anesthesia.

2. **DADLE** - (TyL-D-Ala-Gly-Phe-D-Leu) was purchased from Peninsula Laboratories, Belmont, CA.

DADLE is a biologically stable structural analog of "ENKEPHALIN". ENKEPHALIN means: brain (ENKE) morphine (PHALIN), meaning a morphine-like substance in the brain which is made from naturally existing materials in the brain---amino acids. There are two kinds of naturally-occurring enkephalins, methionine-enkephalin and leucine-enkephalin. The enkephalins, however, are biodegradable. Specific enzymes like enkephalinase and angiotensin converting enzyme can degradate enkephalins. Therefore, enkephalin analogs resistant to enzymatic degradation were synthesized. Many enkephalin analogs resistant to enzymatic degradation were synthesized. Many enzymatically stable enkephalin analogs are available nowadays. Because of the chronological reason, DADLE ([D-Ala^2,D-Leu^5]enkephalin) remains the most known stable enkephalin analog.

For the multiorgan autoperfusion studies, DADLE was infused into intact dogs on the multiorgan bloc at a concentration of 1 mg/kg which is a typical effective dosage for the opioids.

B. ANALYTICAL TECHNIQUES

1. Protein Determinations: Protein content of all resolved plasma fractions will be determined using the method of Lowry et al. (17) as modified by Oyama and Eagle (18).

2. Affinity Chromatography: The affinity chromatography technique, utilizing Affi-Gel Blue (Bio-Rad Industries, Richmond, CA) as the chromatography matrix will be used to rapidly obtain sufficient quantities of nearly homogeneous HIT-containing albumin fractions which will then be subjected to further isolation procedures. This technique has proven highly effective in selectively absorbing albumin from plasma of hibernating or summer-active woodchucks and black bears (19) for experiments in which induction of hibernation in summer-active ground squirrels was used as the bioassay for HIT activity. In this technique carried out at 4°C, the affinity column is loaded with up to 50 mg of lyophilized plasma from hibernating or summer-active animals dissolved in 0.02 M Na-phosphate buffer pH 5.7. A high concentration of salt, 1.4M NaCl, added to the pH 5.7 buffer is then utilized to desorb the HIT-containing albumin fraction from the gel matrix. The column is then regenerated with 2 bed volumes of 6M guanidine HCl.

The homogeneity of 400 ug aliquots of all these fractions is determined by analytical polyacrylamide gel electrophoresis procedure utilizing the standard methodology of Davis (20).

Gels are stained with Coomassi Blue as we have previously described (21). Prior to IV or ICV infusions these fractions are rendered sterile by passing them through a 0.21μ Amicon Sterilet (Amicon, Danvers, MA).

C. IN VITRO CELL CULTURE ASSAY FOR HIT

Since the woodchuck HIT molecule has been demonstrated to alter renal function, (Oeltgen et al. 5), we decided to test the effect of this molecule on a cultured dog kidney cell line (TRMP). This cell line was developed by Dr. Turker and demonstrated to be of epithelial origin (unpublished). In preliminary experiments, serum-starved TRMP cells were stimulated with 5% fetal bovine serum (FBS) in the presence of 0.5 to 2 mg/ml of the woodchuck HIT albumin fraction or the identical albumin fraction from summer-active animals (SAWA fraction). The total number of cells per flask was determined three to four days later.

D. MULTIORGAN AUTOPERFUSION SYSTEM

1. Animals studied

Twenty-one adult mongrel dogs weighing 17 to 30 kg of either sex were used in these studies. The HIT study group consisted of 7 dogs in which hibernation induction trigger(s) (HIT) containing plasma was given before and after the operation. The control group consisted of 8 dogs in which no HIT or DADLE were given.

The DADLE study group consisted of 6 dogs in which DADLE was infused before and after the operation. In order to obtain normal organ wet/dry weight ratio, tissue samples from the heart, lungs, liver, pancreas, duodenum and kidney were taken from ten normal dogs and used for comparison.

2. Pretreatment

All the dogs were given neomycin 2 gm, orally, once a day for three days before the operation in order to sterilize the digestive system. The dogs were fasted 10 hours before the operation. In the HIT study group, 10 ml of plasma containing hibernation induction trigger (HIT), which was obtained by intraventricular puncture from deeply hibernating woodchucks was injected intravenously 2 hours prior to the operation. No HIT was given to the control group. In the DADLE study group, the animals were infused with DADLE at a concentration of 1 mg/kg 2 hours prior to the operation. No DADLE was given to the control group.

3. Surgical Technique

The procedure used to harvest the organs was reported before (11) with the following modifications.

Briefly, the dog was anesthetized with sodium pentobarbital 20 mg/kg, intubated and artificially ventilated. In the supine position, the abdomen was opened and the liver, pancreas, duodenum, both kidneys, abdominal aorta, and inferior vena cava were dissected free. The spleen was removed and one catheter was placed in the splenic artery for arterial blood pressure measurement. Another catheter was placed in the portal vein, via a splenic vein insertion, for blood and fluid transfusion. The common bile duct and the duodenum were cannulated for fluid collections. Two catheters were placed in the ureters for urine collection. The chest was then opened through a medium sternotomy. The diaphragm was incised around the liver. The heart, lungs, aorta, superior and inferior venae cavae (SVC and IVC) were dissected free.

A catheter was placed in the left ventricle (LV) through the carotid artery for LV pressure and dp/dt measurements. Another catheter was placed in the IVC for central venous pressure (CVP) measurement and blood sampling.

The whole system, as shown in Figure 1, was then removed from the body and placed in a water bath containing lactated Ringer's solution, heparin 10 mg/L, neomycin 0.1 g/L and penicillin 100,000 units/L.

4. Interventions

The temperature was maintained around 32°C by heating the water bath with a constant temperature circulator. Artificial respiration was maintained with a Harvard volume-cycled respirator at a tidal volume of 500-700 ml, a rate of 10-20 rpm and PEEP of 2-6 cm H_2O. A gas mixture of 50% O_2 + 3% CO_2 + 47% N_2 was utilized. The following solutions were given at 10 to 20 ml/hour through the portal vein: dextrose (5%), calcium chloride (1 g/L), insulin (50 units/L), mannitol (12.5 g/L), methylprednisolone (500 mg/L), penicillin (1,000,000 units/L), and Flagyl (500 mg/L).

Another 5% dextrose solution containing potassium chloride, 0.5 g, was infused slowly through the portal vein to maintain serum potassium at a normal level.

A fat emulsion (Soyacal) 2 ml, and methylprednisolone 30 mg, were given through the portal vein every 2 hours. Blood transfusions were given to maintain aortic systolic pressure between 75 and 100 mmHg, and CVP between 0 and 10 mmHg. Plasma was given instead of whole blood if the hematocrit was higher than 45%.

5. Application of HIT

Four ml HIT containing plasma, which was obtained from deeply hibernating woodchucks, was given through the portal vein every 4 hours during the preservation period in the study group. Four ml of physiological saline was infused in the control group during the preservation period.

6. Application of DADLE

DADLE at concentration of 1 mg/kg in 1 ml of physiological saline was infused through the portal vein every 24 hours during the preservation period in the study group.

7. Monitoring

Aortic pressure, left ventricular pressure, dp/dt, central venous pressure, portal venous pressure and aortic blood flow were monitored and recorded on a Sensor/Medics R612 Dynograph Recorder throughout the preservation

period. Temperature, urine output, bile production and duodenal and pancreatic secretions were collected and record every hour. Visual changes including color, size and bleeding for each organ, respiratory pressure, tidal volume and PEEP for the lungs were recorded every hour. Arterial blood gas and hematocrit measurements were taken before the operation and every 4 hours during the preservation period and used for blood chemistries, hematology, lactic acid and enzyme measurements for heart, liver, pancreas and kidney functions. Tissue samples were taken from the lungs every 8 hours for tissue wet/dry weight ratio and electron microscope studies. At the termination of the study, specimens were taken from each organ for wet/dry ratios and pathologic examinations.

8. **Determination of Tissue Wet/dry Weight Ratio.**

Tissue samples used for wet/dry ratio measurement were blotted to remove excess fluid and wet weight was measured. The dry weight was determined after the samples had been in an oven at 85°C for 72 hours.

9. **Statistical Analysis.**

All the laboratory tests obtained before the operation (blood gases, hematocrit, blood chemistries, hematology, lactic acid and enzymes for heart, liver, pancreas and kidney functions) were used as normal controls which were compared to the parameters obtained during the preservation period. Heart rate, blood pressures, left ventricular dp/dt, blood gases, urine output, comparisons were made between those obtained immediately after harvesting and those obtained during the preservation period. Tissue wet/dry weight ratios for all the organs were compared with those obtained from the normal control dogs.

Paired and unpaired student's t-tests were used to compare the parameters measured during the preservation period with those obtained preoperatively or immediately postoperatively. The level of significance was 0.05.

RESULTS

1. In Vitro Cell Culture For HIT

Growth reductions ranging from 28 to 67% for the HIT-treated cultures, as compared with the SAWA-treated cultures, were observed. The SAWA treated cultures grew as well or slightly better than cultures which did not receive an Albumin fraction upon serum stimulation. Next, it was determined that the HIT molecule could reduce thymidine incorporation, indicative of a reduction in DNA synthesis, in continuously growing cells. This incorporation assay was also used to determine that when serum starved TRMP cells are stimulated with serum, they entered DNA synthesis approximately 15 hours later and that S-phase lasted for approximately 6-8 hours. The peak of synthetic activity was observed at approximately 18 hours post serum stimulation. Cells stimulated in the presence of 2 mg/ml SAWA fraction demonstrated similar kinetics for re-entering and progressing through S-phase. In contrast, cells stimulated in the presence of 2 mg/ml HIT fraction showed a delayed and reduced amount of DNA synthesis. Significantly, if serum stimulated cells (in the absence of an albumin fraction) are treated with 1-3 mg/ml of the HIT fraction just after they enter S-phase, there is no reduction in DNA synthesis. This result suggests that the HIT molecule effect on DNA synthesis may be effective during only particular times during the cell cycle. Although these results are preliminary, they demonstrate the usefulness of a tissue culture approach for investigations into the mechanism(s) of action of the HIT molecule. Moreover, since the thymidine incorporation assay provides a convenient means for detecting the presence of HIT activity, it will allow for rapid progress in the purification of the HIT molecule.

2. Multiorgan Autoperfusion:

a) The Influence of HIT on Multiorgan Autoperfusion System

The mean survival time of the organs in the HIT-treated study group was 43.4 hours ranging from 33 to 56 hours. Cardiac arrest, resulting from high serum potassium caused "sudden death" in three of the experiments even though all the organs were still in good condition. In the control group, the survival time ranged from 9 to 31 hours with an average of 16.2 hours ($p<0.001$) (Fig. 2A & B)

Cardiac function. In the study group, aortic systolic pressures (AOSP) ranged from 64±2 mmHg to 92±58 mmHg and were easily adjusted by blood or plasma infusion. No inotropic drugs were necessary.

Aortic diastolic pressure ranged from 33±7 to 58±12 mmHg. Aortic pulse pressure ranged from 27±3 to 36±2 mmHg and did not fluctuate appreciably during the preservation period. (Immediately after the operation it was 30±4 mmHg and at 40 hours, it was 28±11 mmHg). CVP ranged from 3.0±1.2 to 8.7±3.2 mmHg. Heart rate ranged from 80±7 to 103±10 beats per minute (Fig 3). Left ventricular maximum dp/dt ranged from 750±353 to 1775±225 mmHg/sec. Calculated maximum dp/dt/p ranged from 11.05±4.60 to 24.14±2.00 (sec^{-1}) (Fig. 4). In the control group, heart function was well maintained during the short preservation period although the survival time was much shorter than the study group.

Heart functions deteriorated 1 to 2 hours before overall failure occurred in the system. If severe infection developed, the heart deteriorated much faster. In the study group, the heart was more sensitive to hyperkalemia. In 3 experiments, sudden heart arrest happened when blood potassium level raised above 6 mmol/L which caused premature death of the whole system even though all other organs were still in good condition.

Lung function. When a gas mixture of 50% O_2 + 3% CO_2 + 47% N_2 was used during the preservation period, arterial oxygen tension (PaO_2) ranged from 180±35 to 285±6 mmHg. Carbon dioxide tension ($PaCO_2$) ranged from 21±2 to 32±4 mmHg. Arterial pH values ranged from 7.28±0.07 to 7.46±0.05 (Fig 5). Lung color changes started earlier than functional changes. However, the lungs maintained good function for more than 40 hours. Lung wet/dry weight ratio was 5.20 at the beginning. It changed to 5.46 at 32 hours and increased to 6.13 at 40 hours.

Liver function. Total bile output during the preservation period ranged from 150 ml to 350 ml with an average of 4.7-5.9 ml/hour. A distinctive difference was found between the study group and control group. The liver began to deteriorate as early as the start of the abdominal operation in the control group. This included swelling, patchy darkening, stiffness to the touch and sweating from the surface. Liver congestion was so severe that sometimes an inotropic drug was necessary in order to maintain a satisfactory arterial blood pressure. The change became worse as the preservation continued. After 12 hours, most of the livers were enlarged, pale and stiff. In the study group, however, the livers had minimal change. In some experiments, they showed signs of congestion, including patchy darkening during the operation. It gradually returned to normal during the preservation period after infusing HIT. Laboratory tests for liver function showed increases in SGOT, SGPT, LDH and ALP immediately after the operation. They decreased during the preservation period and tended to increase again after 36 hours.

The increase was earlier and more severe in the control group (Fig. 6,7).

Pancreatic and duodenal function. The pancreas and duodenum had minimal changes during the preservation period. Secretions from these organs ranged from 3-9 ml/hour (total 130 to 450 ml). Blood amylase levels did not change during the preservation period (Fig. 8).

Renal function. Total urine output ranged from 800 to 2400 ml during the preservation period with hourly urine output averaging from 15 to 70 ml in the study group. In the control group, total urine output ranged from 13 to 650 ml with an average of 0.6 to 54 ml per hour. Kidney function was well preserved during the preservation period in the study group. Blood urea nitrogen (BUN) was reduced to about 40% of the starting levels during the preservation period ($p<0.0005$ at 24 hours). Blood creatinine levels decreased by 70% at 8 hours ($p<0.025$) and maintained a significantly lower level throughout the experiments ($p<0.025$ at 24 hours) (Fig. 9). In the control group, premature renal failure occurred in two experiments. Both BUN and creatinine also decreased while the kidney was working.

Hematology study. Stable RBC concentrations were maintained during preservation by blood or plasma transfusions. Heparin was not used therefore bleeding was not a big concern even though the dissection was extensive. RBC concentrations tended to increase due to the exudation of lymph. Plasma infusion was always necessary in all experiments to keep hematocrit at normal levels. WBC counts had a continuous decrease during the preservation period in both groups ($p<0.0005$ at 12 and 24 hours). Blood platelet levels had slight decreases during the preservation period but was not statistically significant (Fig. 10). Free plasma hemoglobin had only a two-fold increase at 24 hours in the study group. The increase was severe in the control group (Fig. 11).

Blood chemistry. Serum potassium, calcium and glucose were replaced as needed to maintain normal levels. Serum potassium tended to decrease due to high volume urine output. However, a relatively lower serum potassium did not cause much trouble. Sodium and chloride levels remained in the normal range during the preservation period in both groups (Fig. 12).

Pathology study. Lung tissue samples were studied by electron microscope and good tissue preservation up to 40 hours was revealed. There was moderate interstitial widening caused by edema at 40 hours. Good tissue preservation was also noted from the heart, liver, pancreas, duodenum and kidney at 36-44 hours.

Lung transplantation. In the study group, an acute lung transplantation was performed after 33 hours of preservation time. An adult dog weighing 23 kg which matched the donor dog was used as the recipient. The dog was anesthetized with sodium pentobarbital, 30 mg/kg, intubated and artificially ventilated. A left thoracotomy was made through the 5th intercostal space and the left lung was removed. In the preservation system, the left lung was taken down and transplanted in standard fashion (22). The right pulmonary artery was ligated immediately after the transplantation. The dog was maintained on anesthetic and artificially ventilated for 12 hours with 100% oxygen used for ventilation. Blood pressures and blood gases were monitored and were maintained at satisfactory levels after transplantation. (Fig. 13).

b. The Influence of DADLE on the Multiorgan Autoperfusion System

Our prior work utilizing a ground squirrel bioassay model had shown that DADLE was the only opiate molecule capable of inducing hibernation in summer-active ground squirrels in a fashion similar to that produced by injection of 10 mg (5 mg/protein) of the HIT-containing albumin fraction (9). We therefore decided to see if this delta opiate would also mimic the action of the HIT-containing plasma in effectively extending organ preservation time utilizing the multiorgan autoperfusion system. In the study group of 6 dogs, 25 mg of DADLE (Penninsula Laboratories, Belmont, CA) was injected I.V. into the intact dog 60 and 30 minutes prior to harvesting of organs for the autoperfusion system. In the organ bloc, 7.5 mg of DADLE was injected through the portal vein every 2 hours during the preservation period. The dose of DADLE given to the intact dog and the multiorgan preparation approximates a concentration of 1 mg/kg which has been commonly used and reported in the opiate literature. All parameters were kept identical in the previously described HIT study. The organ system survival time ranged from 41 to 60 hours with an average of 46.7 hours. (Figs. 14A and B). Similar to the HIT studies cardiac lung, renal, pancreatic, hepatic, hematological and blood chemistry function were well maintained compared to controls. In the DADLE-treated group, aortic systolic pressures ranged from 62±6 mmHg to 79±53 mmHg and were easily adjusted by blood or plasma infusion. No inotropic drugs were necessary. Aortic diastolic pressure ranged from 33±44 to 49±7 mmHg. Aortic pulse pressure ranged from 23±4 to 30±6 mmHg and did not fluctuate appreciably during the preservation period. CVP ranged from 4.9±0.6 to 9.2±2.9 mmHg. Heart rate ranged from 86±9 to 100±12 beats per minute. Left ventricular maximum dp/dt ranged from 1250±353 to 1920±163 mmHg/sec. Calculated maximum dp/dt/p ranged from 17.2±2.3 to 25.4±4.5 (sec^{-1}).

Arterial oxygen tension ($Pa0_2$) ranged from 264±32 to 348±9 mmHg. Carbon dioxide tension ($Pa/c0_2$) ranged from 13.6±2.4 to 23.8±6.1 mmHg. Arterial pH values ranged from 7.29±0.07 to 7.48±0.08 Lung wet/dry weight ratios were 5.40 at the beginning. It changed to 5.81 at 24 hours and increased to 6.35 at 40 hours. Total bile output during the preservation period ranged from 122 ml to 300 ml with an average of 2.7-6.1 ml/hour. In the control group, total urine output ranged from 13 to 800 ml (0.6-59 ml/hour). In the study group, blood urea nitrogen reduced from 13.86 to 6.17 mg/dL at 44 hour. Blood creatinine levels decreased from 1.04 to 0.65 mg/dL at 44 hours. Laboratory tests for liver function showed increases in SGOT, SGPT, LDH and ALP immediately after the operation. They decreased during the preservation period and tended to increase again after 36 hours in the study group. The pancreas and duodenum had minimal changes during the preservation period. Secretions from these organs ranged from 1.4-8.9 ml/hour (total 60-500 ml). Blood amylase levels maintained stable during the preservation period. Total urine output ranged from 1600 to 2500 ml during the preservation period, (28-60 ml/hour) in the study groups. No premature renal failure occurred in the study group. RBC concentrations were maintained stable during preservation by blood or plasma transfusions. Plasma infusion was always necessary in all experiments to keep hematocrit at normal levels. WBC counts had a continuous decrease during the preservation period in both groups ($p<0.0001$ at 44 hours). Blood platelet levels had slight decrease during the preservation period ($p<0.01$ at 40 hours). Serum potassium, calcium and glucose were replaced as needed to maintain normal levels. Serum potassium tended to decrease due to high volume urine output. However, a relatively lower serum potassium did not cause much trouble. Sodium and chloride levels remained in the normal range during the preservation period is both groups. Unlike the control study, no severe liver congestion occurred or premature renal failure occurred and lung function was good up to more than 55 hours in the longest survival experiment. These studies clearly indicated that DADLE was comparable to HIT in effectively extending organ survival tie of the multiorgan autoperfusion system.

3. POSSIBLE MECHANISM BY WHICH HIT EXERTS ITS ACTION

The autoperfusion methodology had extended organ survival time, however, progressive organ failure would still occur after some time. Many factors can affect the overall survival time. Surgical trauma, ischemia during preservation, accumulation of toxic substances and formation of circulating platelet aggregates all have a negative effect on tissue survival. One feature that was a consistent finding in all of our studies was the

progressive fall in the white cell and platelet counts during the course of the experiment. Although histologic studies are incomplete because of the sheer volume of material to be examined, serial lung sections showed clumps of white cells and platelet aggregates in the vascular space. These two findings suggest that the hypothesis that one reason for progressive organ dysfunction during the autoperfusion studies may result from embolization of platelet and neutrophil aggregates in the heart, lung, liver and kidney.

In the canine model, the occurrence of circulating platelet and neutrophil aggregates can be detected by ultrasound. Therefore, it is of interest to perform serial ultrasonic studies during the period of autoperfusion to determine if these are changes from normal in ultrasonic data. The inferior vena cava contains particles that move white blood flow and appear to increase in size and number during the course of the autoperfusion study. The ultrasonic detection of large numbers of particles during cardiac surgery as a result of cardiopulmonary bypass has previously been associated with multiorgan failure and poor prognosis.

In preliminary studies, the ultrasonic appearance to the blood may be a biomarker for impending organ failure due to the accumulation of embolic material. Although the microscopic appearance of the peripheral blood during the course of these experiments appears to suggest and association between the presence to platelet and neutrophil aggregates and the duration of survival, too few animals have been studied to draw a definitive conclusion.

If the embolization of platelet and neutrophil aggregates cause progressive organ failure in the autoperfusion model, and the survival or organs is augmented by the use of HIT, one would hypothesize that one effect of HIT is to alter platelet function. This was studied in one dog prior to the following intravenous administration of HIT. In the canine model, platelets behaved normally prior to HIT administration, with a dose response relationship between the amount of adenosine diphosphate (ADP) added and the extent of aggregation. However, after HIT, even though the platelets aggregate normally in response to adp, the platelets disaggregated shortly after ADP stimulated aggregation, despite the use of high dose ADP, as shown in the Figure 15. It has also been reported that human erythocytes could agglutinate in human serum at low temperatures due to the presence of cold agglutinins. However, RBC did not agglutinate when placed in hibernator's serum. These results suggest that one mechanism of prolonged organ survival following HIT administration may be a result of its ability to reverse cell aggregation. We will conduct an entire series of experiments using HIT to verify this initial observation.

DISCUSSION

The slow progress in defining the chemical identify and fully characterizing the HIT molecules(s) may primarily be attributed to the necessity of utilizing a bioassay requiring induction of hibernation in summer-active ground squirrels (a very restrictive seasonal time frame for testing). We now report the first success in preliminary experiments in developing a rapid in vitro cell culture bioassay system which can be utilized year round for demonstrating HIT-activity of more highly resolved plasma fractions. Moreover, this bioassay system may ultimately be utilized for determining the mechanism(s) by which HIT and the delta opioid. DADLE, act at the cellular level and may ultimately allow us to clone the HIT fraction genes and its mRNA.

Our primate experiments have clearly demonstrated that hibernators are not unique in their ability to respond dramatically to the introduction of the opioid-like HIT molecule and indicated that the clinical potential for such a profound metabolic inhibitor and its related compounds may be vast. It therefore seemed reasonable for us to investigate whether the HIT molecule and/or DADLE, could effectively extend tissue survival time in a multiorgan autoperfusion system developed by Dr. Sufan Chien. The multiorgan autoperfusion technique has the following advantages: 1. It has no ischemic time from removing the organs to preserving them in vitro. This is especially important for such organs as the heart, lungs and liver because they are very vulnerable to ischemia. 2. It retains a natural circulation. A circulatory volume is large enough so that no foreign material is necessary to help the circulation. As a result: A. No anticoagulation (heparin) is necessary. B. Blood circulates in a natural vascular tree so that hemolysis is minimal. 3. It is a self-contained organ block with a relatively complete physiologic environment. Metabolic wastes are removed and water and electrolyte balance is maintained automatically by the kidneys and major metabolic processes are taken care of by the liver. 4. The preparation is relatively simple. A respirator is the only necessary commercial equipment. 5. Because of the organs are outside the body, physiologic, pharmacologic and pathologic studies are convenient.

Our study utilizing the opioid-like HIT molecule and the delta opioid DADLE to effectively extend organ preservation time using the multiorgan autoperfusion system will allow us to monitor the activity of variously resolved plasma fractions for HIT activity. Moreover, a number of clinically relevant uses for HIT and DADLE may be explored utilizing this autoperfusion system.
These include the following: 1.) Establishing a totally new technique for organ preservation and transplantation.

2.) Documenting that the opioid-like HIT molecule and the delta opioid, DADLE can markedly extend effective organ preservation time, and 3.) Defining the mechanisms by which the HIT and DADLE extend organ survival time. The latter study may confirm the role of these molecules in preventing platelet and/or platelet-neutrophil aggregation phenomena which may result in microthromboemboli which can have widespread deleterious effects on the microvasculature during organ perfusions and limits on organ survival following any major vascular surgical procedure.

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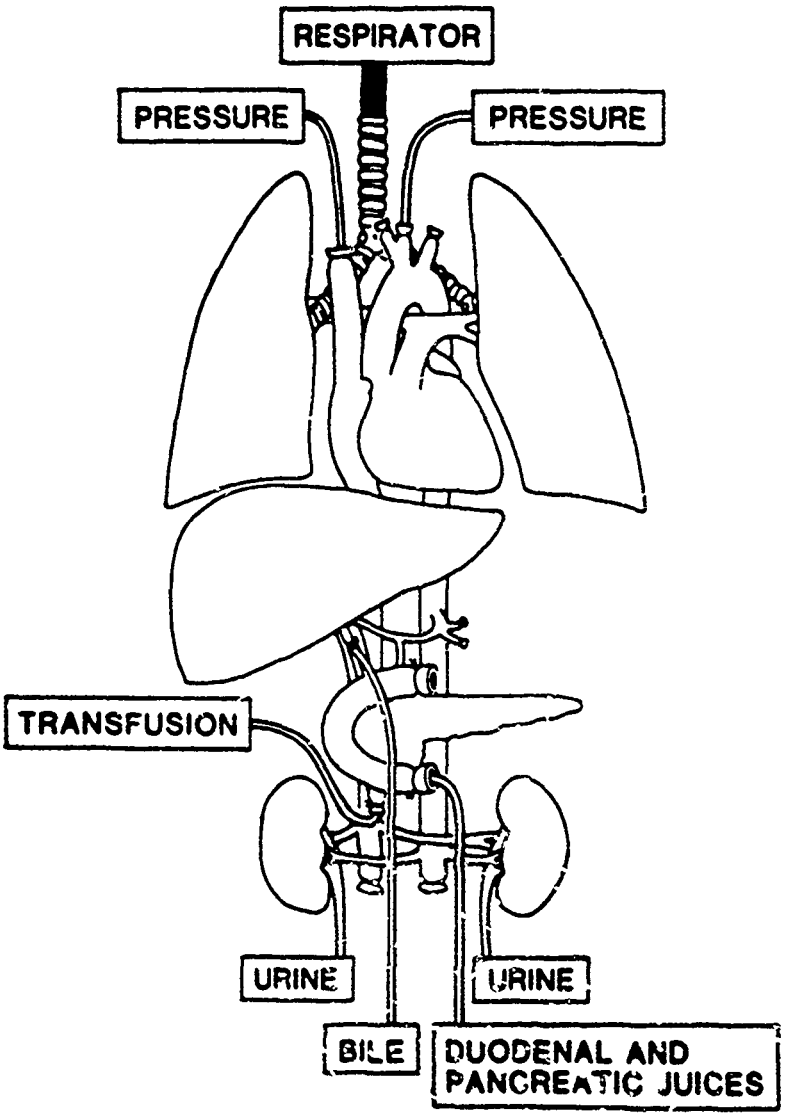

Fig. 1. Simultaneous multiorgan autoperfusion preservation in which the heart, lungs, liver, pancreas, duodenum and two kidneys are preserved in one system. No foreign material is used to assist the circulation.

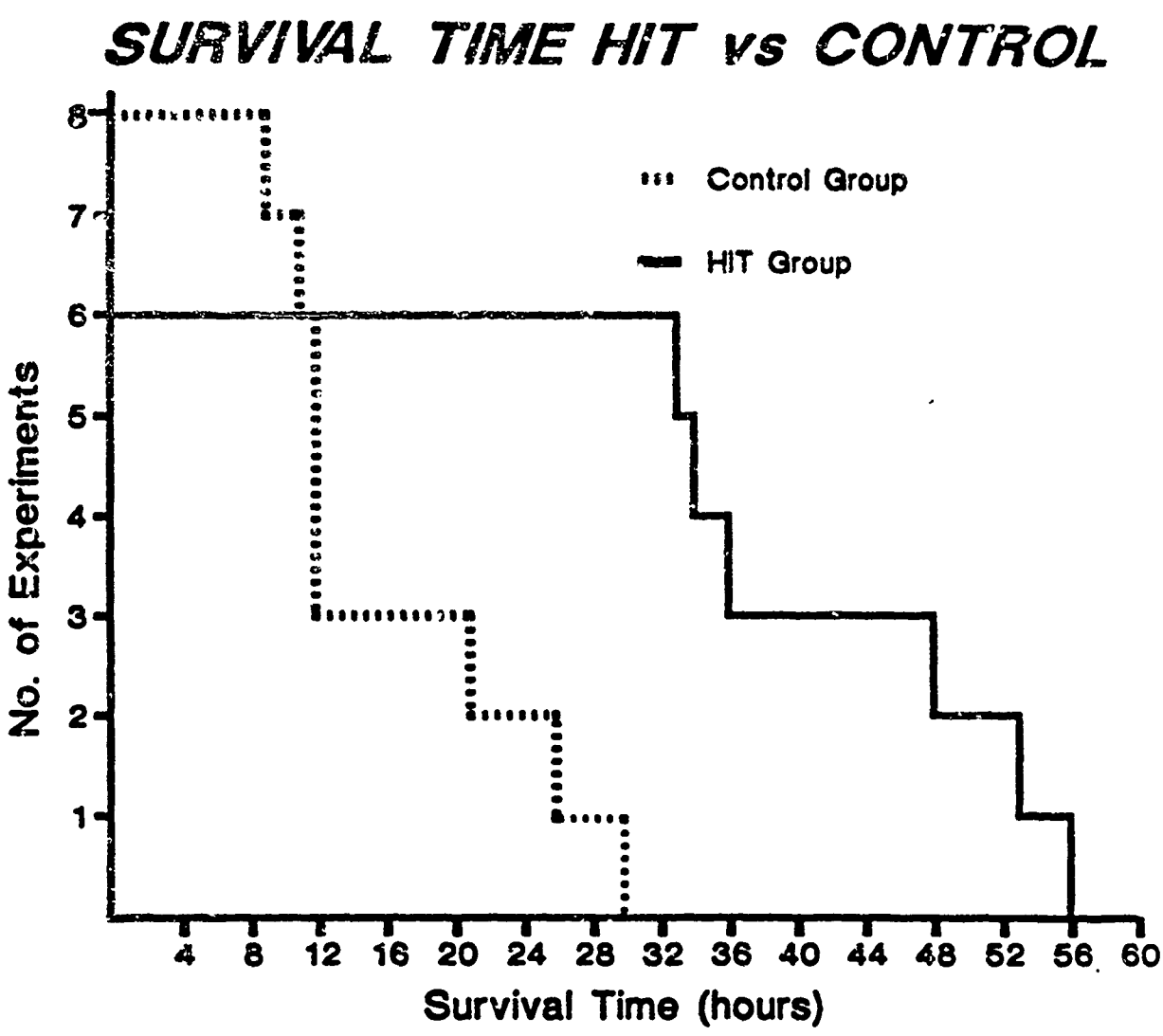

FIGURE 2A. Survival of individual multiorgan preparations in the control group (n=8) versus the HIT treated group (n=6).

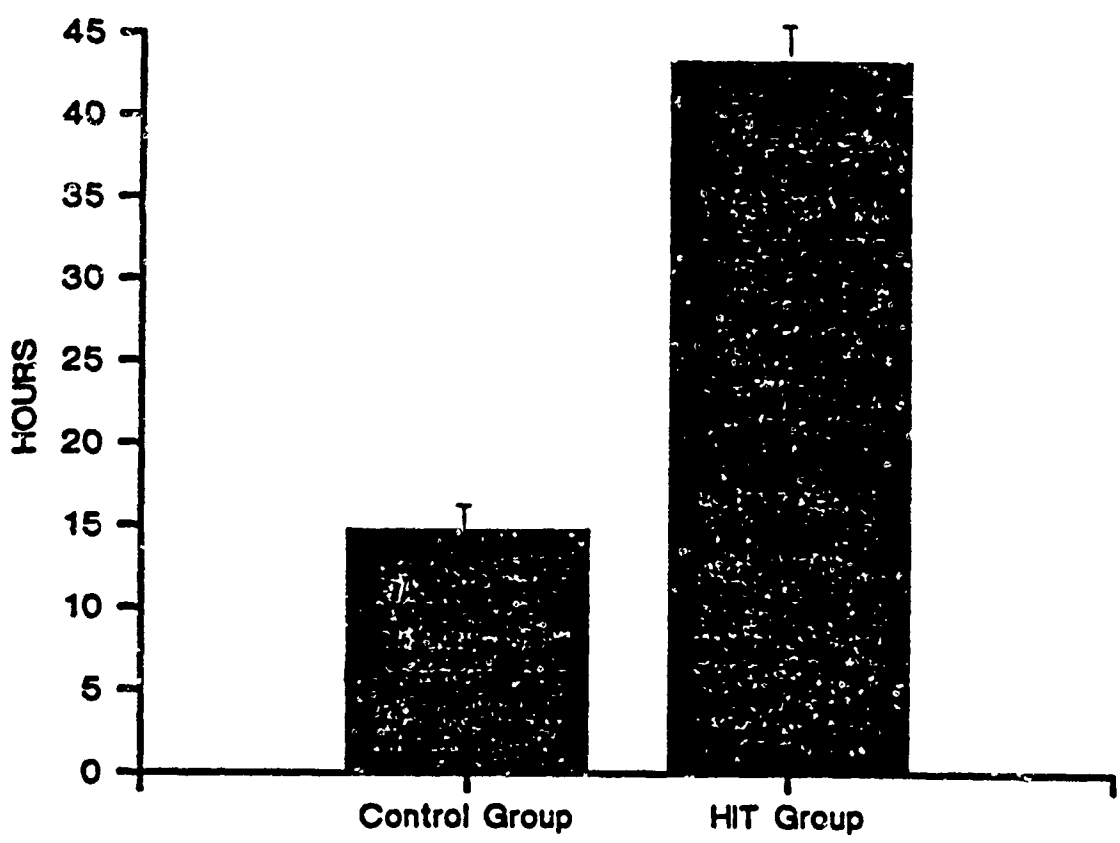

FIGURE 2B. Survival time in the study group with HIT and the control group without using HIT ($p<0.00025$). Three early deaths occurring between 33-36 hours in the study group were caused by hyperkalemia.

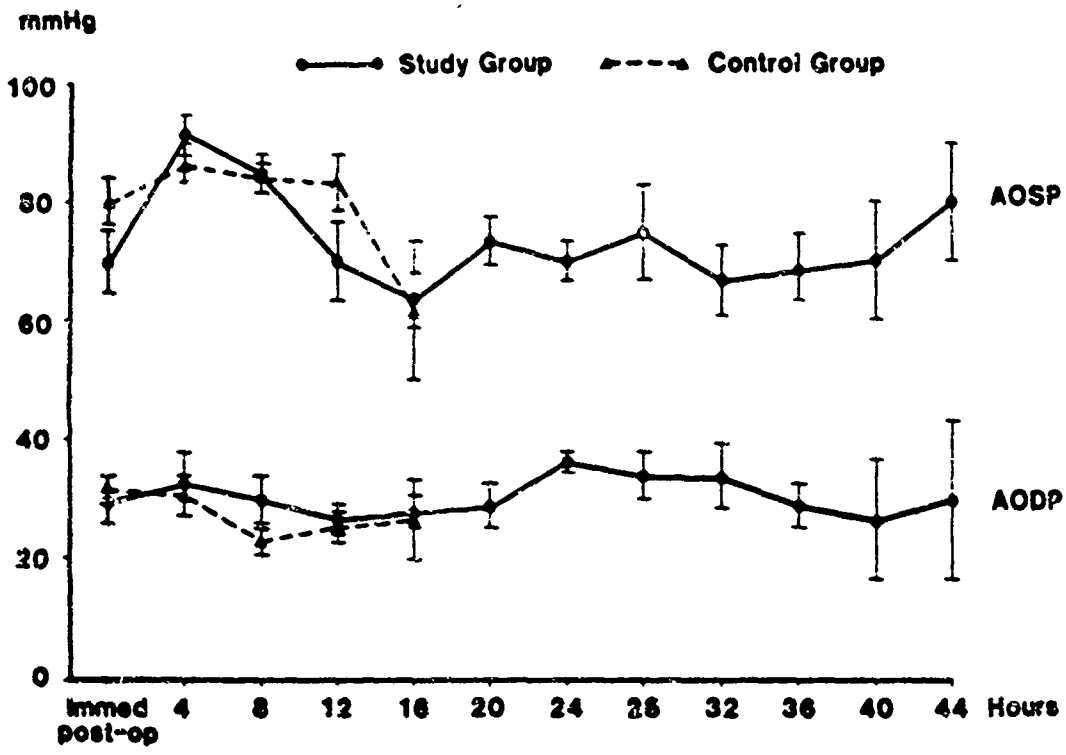

Fig. 3. Aortic systolic pressure (AOSP) and diastolic pressure (AODP) during the preservation period.

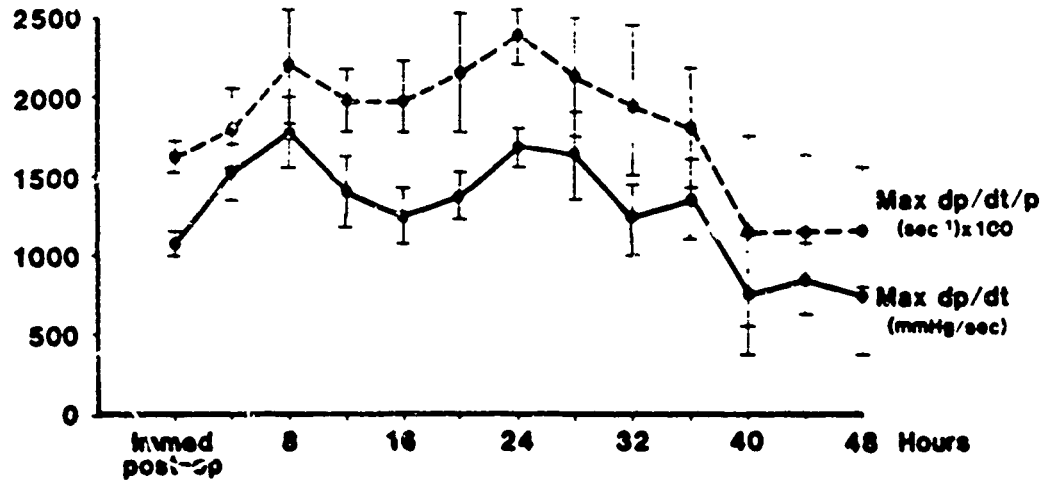

Fig. 4. Changes of left ventricular maximum dp/dt and dp/dt/p during the preservation period in the study group.

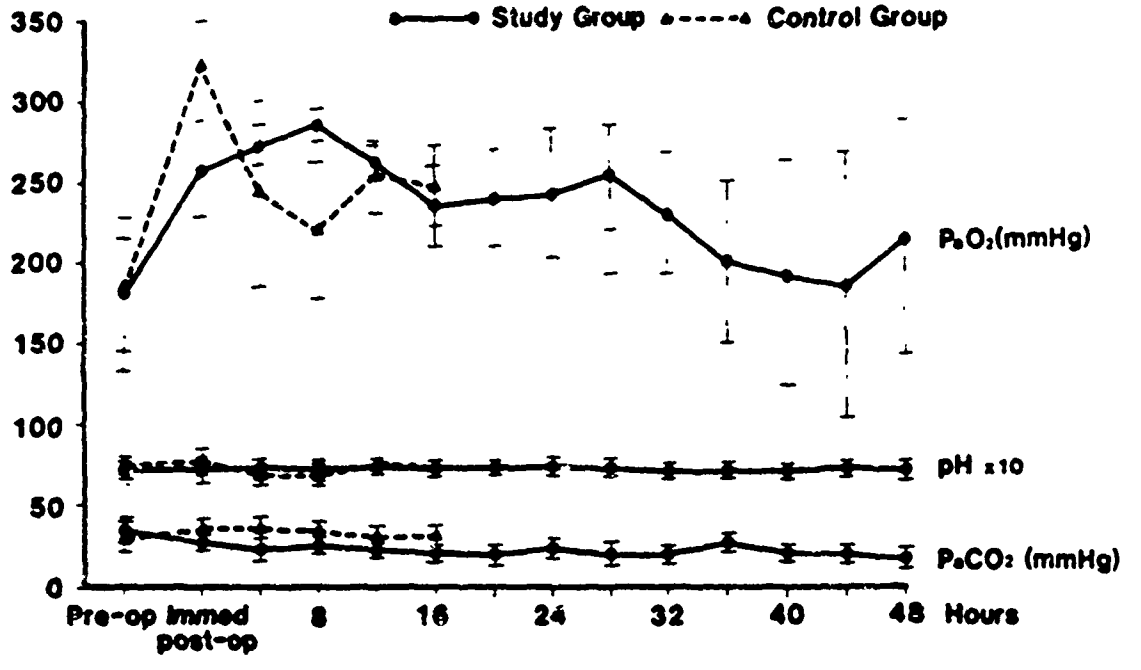

Fig. 5. Change of blood gas values during the preservation period. P_aO_2: Arterial oxygen tension. P_aCO_2: Arterial carbon dioxide tension.

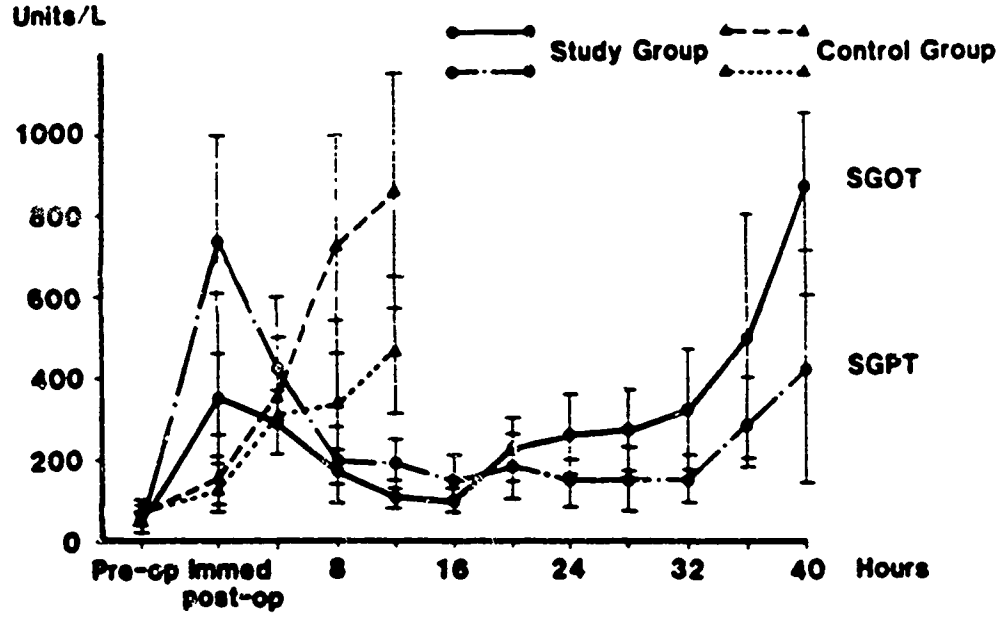

Fig. 6. Serum glutamic-oxalacetic transaminase (SGOT) and glutamic-pyruvate transaminase (SGPT) during the preservation period.

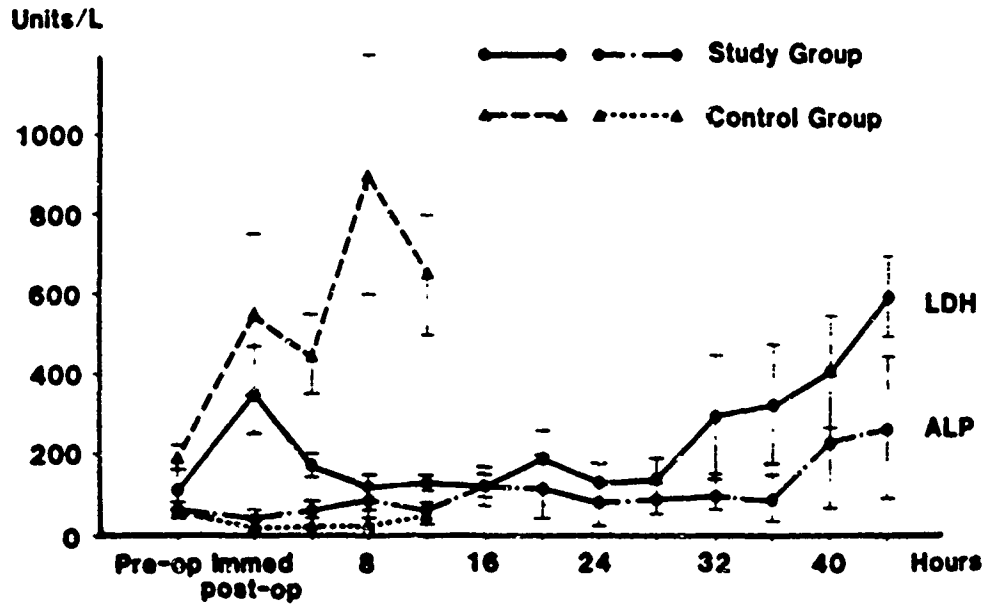

Fig. 7. Serum alkaline phosphatase (ALP) and lactic dehydrogenase (LDH) during the preservation period.

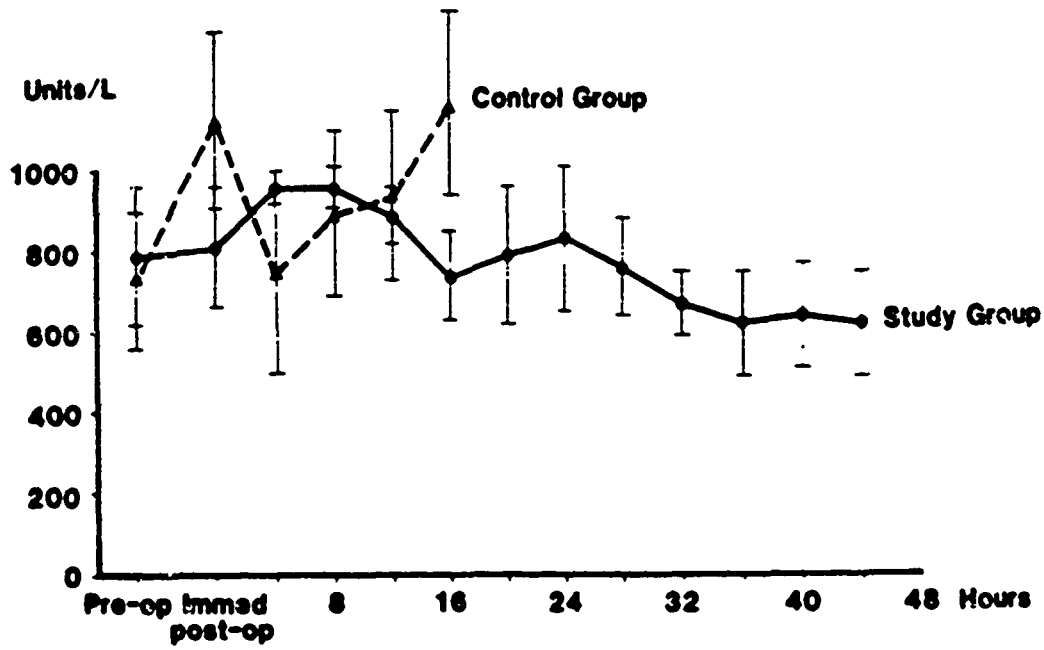

FiG. 8. Changes of blood amylase levels during the preservation period.

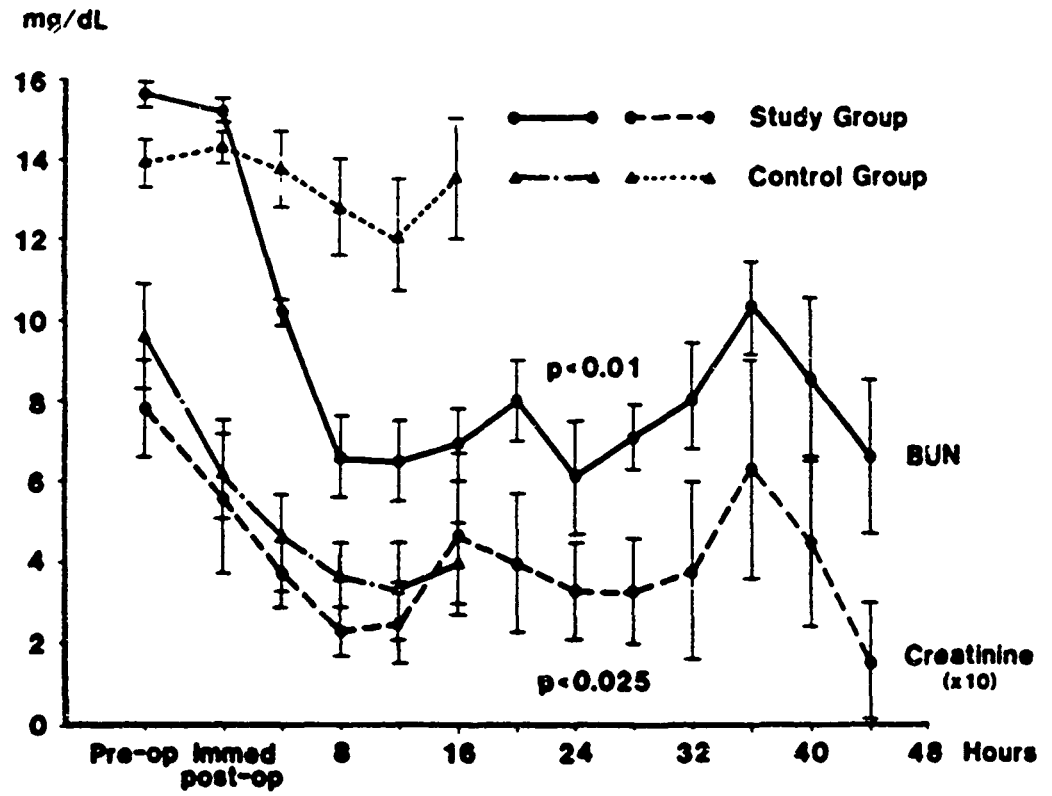

Fig. 9. Changes of blood urea nitrogen (BUN) and creatinine levels during 44-hour period. (All p values are the comparisons between pre-op levels and those obtained during the preservation period).

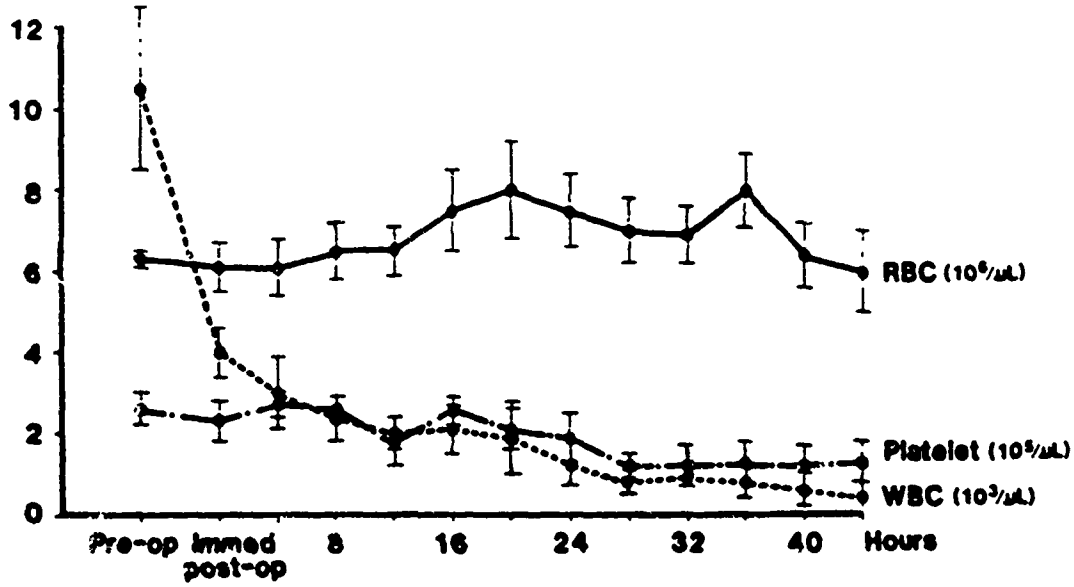

Fig. 10. Changes of blood cells during preservation. RBC: red blood cell, WBC: white blood cell.

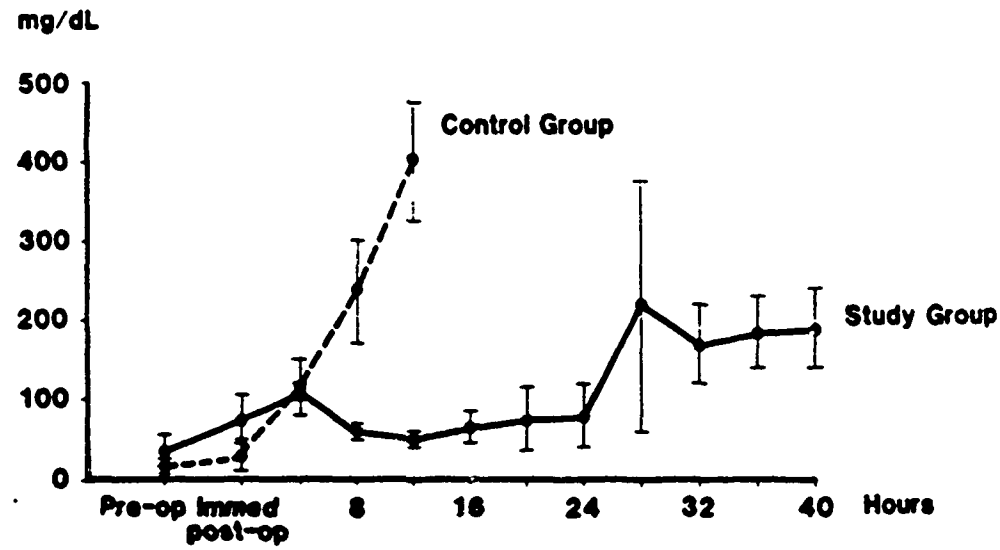

Fig. 11. Change of plasma free hemoglobin during the preservation period.

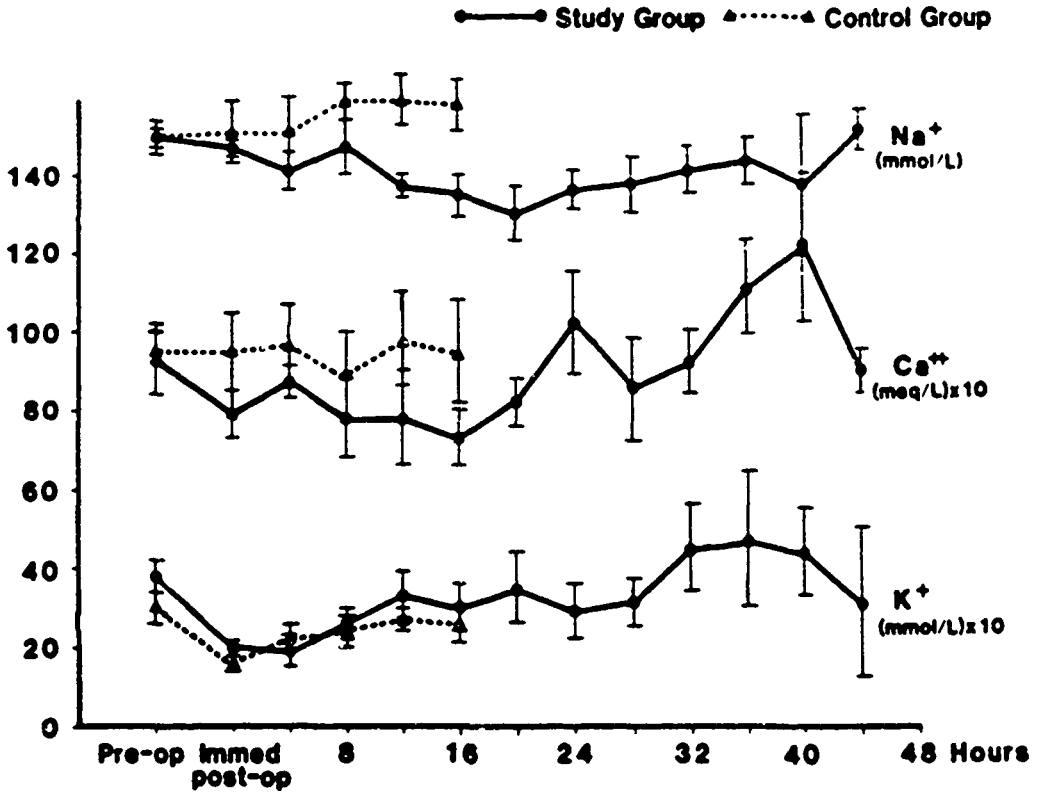

Fig. 12. Changes of serum potassium (K^+), sodium (Na^+), and calcium (Ca^{++}) during preservation.

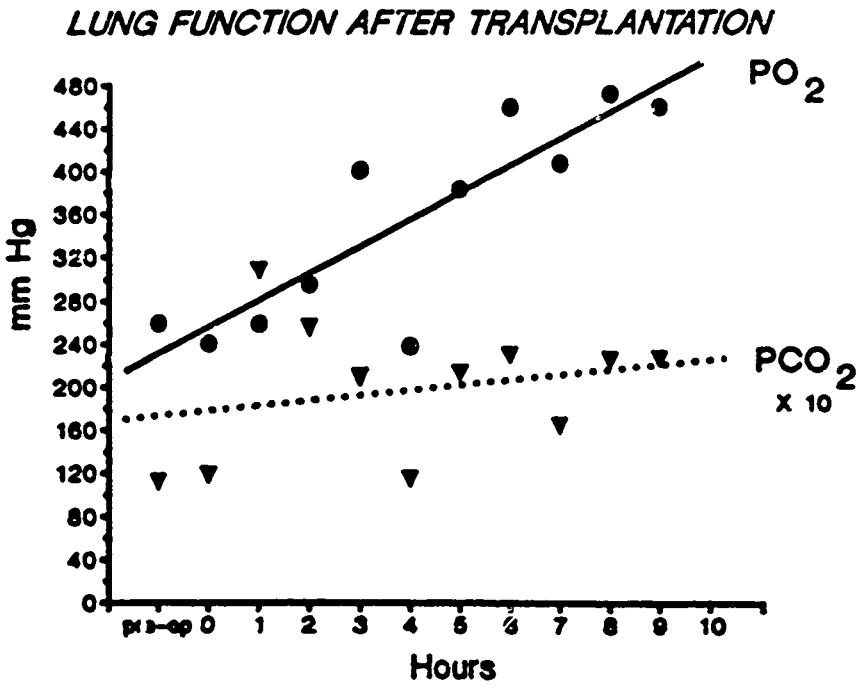

Fig. 13. Arterial blood gases after left lung transplantation.

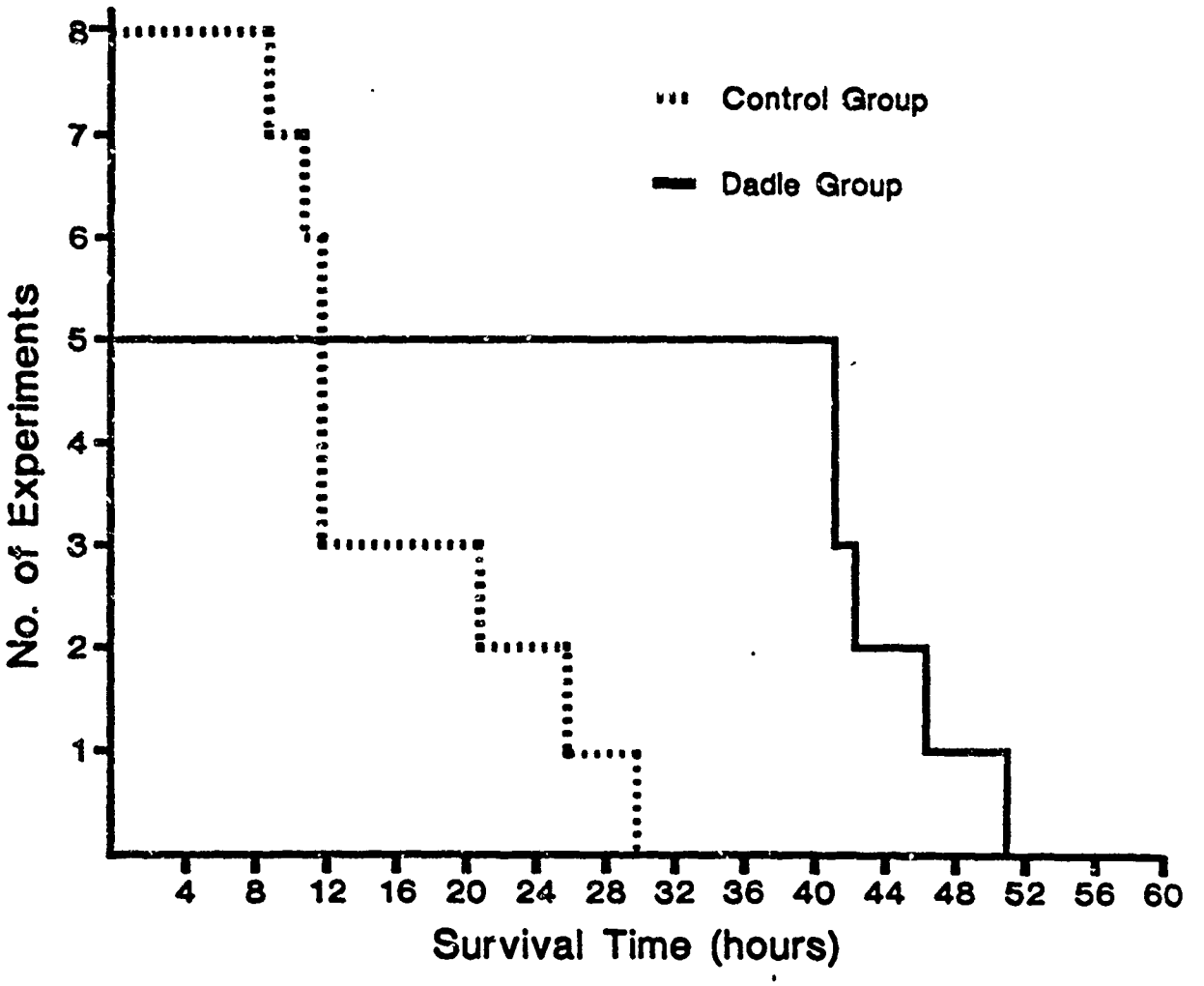

FIGURE 14A. Survival of individual multiorgan preparations in the control group (n=8) versus the DADLE-treated group (n=5).

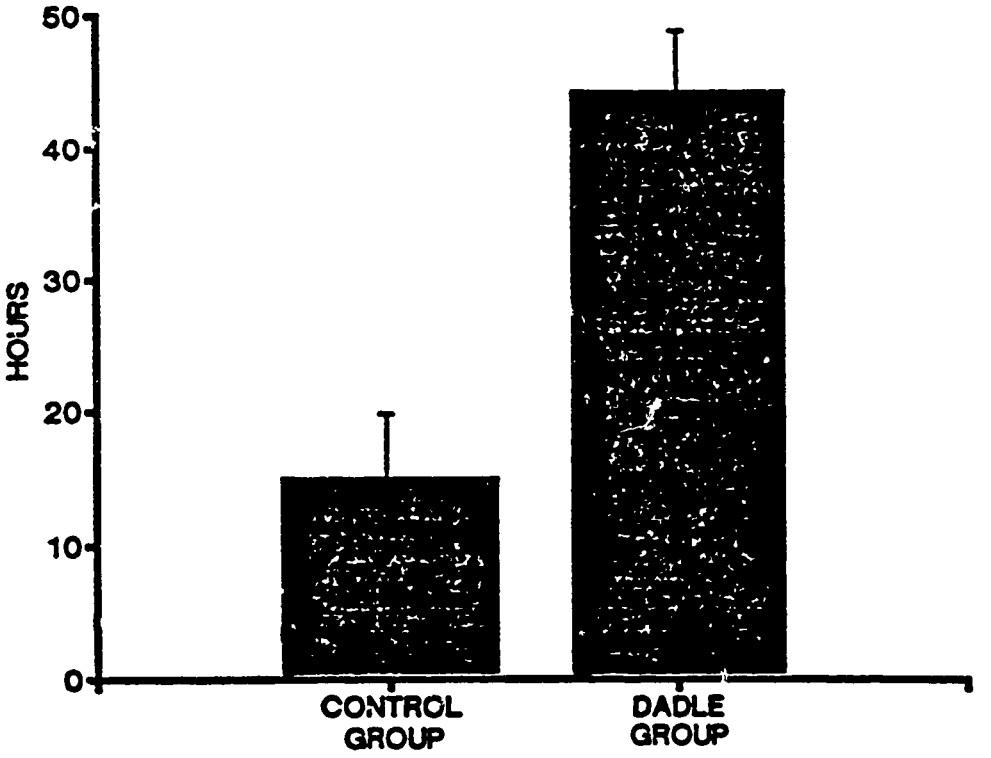

FIGURE 148. Comparison of survival time between the study group using DADLE and the control group without using DADLE ($p<0.0001$).

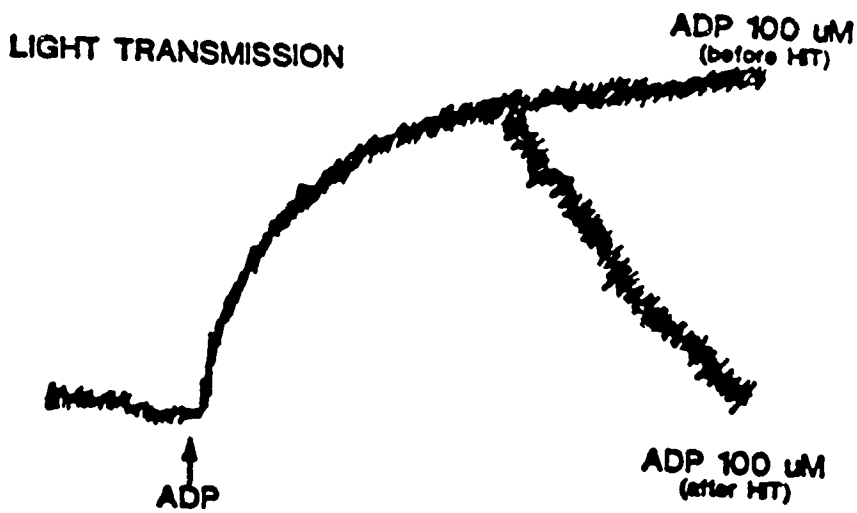

FIGURE 15. Platelet diaggregation following HIT administration despite high dose ADP to stimulate aggregation. Platelets behaved normally prior to HIT administration, with a dose response relationship between the amount of adenosine diphosphate (ADP) added and the extent of aggregation. However, after HIT, even though the platelets aggregate normally in response to ADP, the platelets disaggregated shortly after ADP stimulated aggregation.

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